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Novel Real-Time PCR Assay for Detection of *Helicobacter pylori* Infection and Simultaneous Clarithromycin Susceptibility Testing of Stool and Biopsy Specimens

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A biprobe real-time PCR protocol, followed by hybridization melting point analysis, to detect point mutations in the 23S rRNA gene of *Helicobacter pylori* associated with clarithromycin resistance was established and evaluated in a clinical study. Of 92 patients who underwent endoscopy, 45 were found to be *H. pylori* infected and invariably were also culture positive. Of the 45 isolates, 11 were shown to be resistant to clarithromycin by E-test. With respect to the detection of *H. pylori* infection, PCR showed sensitivities of 100% in biopsies and 98% in stool specimens and a specificity of 98% in both biopsy and stool samples. All clarithromycin-sensitive cases were identified as such by PCR in both biopsy and stool samples. Of the cases with a resistant strain, eight were identified as such in stool DNA and nine were identified in biopsy DNA. Failure of PCR to detect the resistant genotype in the biopsy DNA, stool DNA, or both (one case) was associated with mixed populations. In these cases, patients had not been treated for *H. pylori* infection before, and the sensitive population showed to be present in considerably higher numbers than the resistant population. In five of six cases in which infection with a resistant genotype only was identified by PCR, the patients had received clarithromycin-based eradication therapy in the past. Thus, the assay presented provides a highly accurate noninvasive method to detect *H. pylori* infection in stool and at the same time allows for culture-independent clarithromycin susceptibility testing.

Helicobacter pylori is a gram-negative bacterium associated with different digestive diseases, such as gastritis, peptic ulcer, mucosa-associated lymphoid tissue lymphoma, and gastric cancer (3). At present, several diagnostic assays for *H. pylori* detection are available (23). Invasive methods requiring gastric endoscopy include rapid urease testing, culture, histology, and molecular diagnostics. Noninvasive approaches include fecal antigen detection, serologic testing, and urea breath testing. During recent years, noninvasive methods gained in importance; however, no information on resistance against antibiotics can yet be obtained with these tests. Clarithromycin is an integral part of first-line therapies to treat *H. pylori* infection. As demonstrated by a meta-analysis of published data, susceptibility or resistance to clarithromycin result in eradication rates of 81 to 95% and 0 to 48%, respectively (8). Since clarithromycin is a widely used antimicrobial drug, the prevalence of clarithromycin-resistant *H. pylori* strains is increasing continuously.

Resistance of *H. pylori* to clarithromycin is mainly due to an adenine-to-guanine transition at positions 2142 and 2143 and to an adenine-to-cytosine transversion at position 2142, which are included in the peptidyltransferase loop of the 23S rRNA (24). Recently, several PCR based methods, such as PCR-restriction fragment length polymorphism (17), PCR-DNA-enzyme immunoassay (13), reverse hybridization line probe assay (22), and real-time PCR methods combined with melting curve analysis by biprobes and hyprobes (2, 5, 10, 14, 18), were performed with cultured strains or biopsies in order to determine susceptibility to clarithromycin. However, clarithromycin susceptibility testing by PCR of stool samples would be more rapid and convenient, eliminating the need for gastric endoscopy.

Thus, the aim of the present study was to develop a real-time PCR hybridization assay that can be used for both specific detection of *H. pylori* infection and for the determination of point mutations in the 23S rRNA gene responsible for clarithromycin resistance by using biprobe technology. Biprobes are sequence-specific probes labeled with the fluorophore Cy5. When the probe hybridizes to the target sequence, Cy5 is excited by the energy transfer from SybrGreen I, resulting in an increase of emitted light. In the presence of mismatched bases between the probe and the target, melting curve analysis reveals a lower melting temperature than in the case of a perfectly matched sequence (2, 5). This test should then be evaluated in a clinical study by using both gastric biopsy and stool specimens. With respect to the detection of *H. pylori* infection, the biprobe assay was also compared to a novel TaqMan real-time PCR assay for the detection of *H. pylori*-specific *ureA*.

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MATERIALS AND METHODS

Patients, samples, and bacterial strains. Fecal and biopsy samples were collected from 92 adult patients (age range, 19 to 90 years) suffering from abdom-

inal pain in the Ambulatory Care Centre South, Regional Public Medical Insurance Agency, Vienna, Austria. Of these, 18 patients had previously received antibiotic treatment for *H. pylori* infection. Gastric biopsy samples were subjected to histology, rapid urease test, and culture. For culture, biopsy samples (both antrum and corpus) were transported in Portagerm Pylori Transport Medium (bioMerieux, Marcy l'Etoile, France) and homogenized in 1 ml of 0.9% NaCl. Of the homogenate, 450 μl were plated on Pylori agar (bioMerieux) and incubated up to 10 days to selectively culture *H. pylori*. Clarithromycin susceptibility of the isolates was tested by E-Test (AB Biodisk, Solna, Sweden) on Mueller-Hinton blood agar (Heipha Dr. Müller GmbH, Heidelberg, Germany). A strain was considered resistant to clarithromycin when the MIC was ≥1.0 μg/ml (16). Cultures were performed at 37°C under microaerobic conditions. Patients were considered to be noninfected when found to be negative for *H. pylori* by histology, rapid urease test, and culture. They were considered to be infected when found to be positive for *H. pylori* by both histology and rapid urease test or by culture alone. Samples from patients found to be positive by either rapid urease test or histology alone were also used for further testing by real-time PCR; however, the results were not considered for statistical evaluations.

Stool specimens and biopsy homogenates were stored at −70°C until they were used for DNA extraction. From biopsy homogenates (100 μl), DNA was extracted by the QIAamp DNA minikit by using the blood and body fluid protocol (Qiagen, Hilden, Germany). From stool specimens (0.2 g), DNA was extracted by using the QIAamp DNA stool minikit (Qiagen). In order to increase the yield of purified DNA, the whole supernatant (500 μl) was used for further DNA purification after the addition of the InhibitEX tablet, adapting the following steps of the Qiagen protocol to this altered sample amount. From *H. pylori* isolates, DNA was extracted by using the QIAamp DNA minikit according to the protocol for isolation of genomic DNA from bacterial cultures (Qiagen). DNA extracts of *H. pylori* reference strain CCUG 38771 with the wild-type 23S rRNA genotype and strains 825, 683, and 677 harboring the known mutations A2142C, A2142G, and A2143G, respectively, in the 23S rRNA gene were used as positive controls (18).

Real-time PCR. GenBank was searched for sequences of the genes encoding *ureA* and 23S rRNA of *H. pylori*. The published sequences were aligned by using CLUSTAL W (http://www.ebi.ac.uk/clustalw/), and primers and probes were designed by using Primer Express software (Perkin-Elmer/Applied Biosystems, Foster City, Calif.) and the LightCycler probe design software (Roche Diagnostics, Mannheim, Germany). A BLAST search was performed to check the specificity of the DNA sequences of the primers and probes (http://www.ncbi.nlm.nih.gov/BLAST/). Primers and probes for *ureA* were selected from a region with 100% homology between all published *H. pylori ureA* encoding sequences. The BLAST search revealed 100% homology to the *H. pylori*-specific *ureA* gene only. Primers for the 23S rRNA gene were selected from a region as close as possible to the mutation sites. Primer 23S-F showed 100% homology to *H. pylori*, *H. hepaticus*, *H. acinonychis*, *Arcobacter cryaerophilus*, and *Wolinella succinogenes* and to some of *Campylobacter jejuni* published sequences. Primer 23S-R showed 100% homology to *H. pylori* and *H. acinonychis* only. Five mismatches were found in the corresponding sequence of *A. cryaerophilus*, and three were found in those of *H. hepaticus*, *W. succinogenes*, and *C. jejuni* each. Probe 23S-S showed 100% homology to a greater number of bacterial genera. Therefore, the specificity of this assay was mainly provided by the primers. Stool and biopsy DNA extracts were subjected to real-time PCR, which was performed in a LightCycler apparatus (Roche Diagnostics). Samples were run in duplicate and were considered positive if at least one of the reactions was positive.

For *ureA* detection (77-bp PCR fragment), the 20-μl reaction mixture was prepared as follows: 2 μl of LightCycler-FastStart DNA Master Hybridization Probes (Roche Molecular Biochemicals, Mannheim, Germany), 4 mM $MgCl_2$, 0.25 μM primer ureA-F (5′-CGTGGCAAGCATGATCCAT-3′, positions 2877 to 2895, GenBank accession no. M60398), 0.25 μM primer ureA-R (5′-GGGT ATGCACGGTTACGAGTTT-3′, positions 2953 to 2932, GenBank accession no. M60398), 0.2 μM TaqMan probe ureA-S (5′-Fam-TCAGGAAACATCGC TTCAATACCCACTT-Tamra-3′, positions 2924 to 2897, GenBank accession no. M60398), and 2 μl of DNA extract made up to 20 μl with water. The amplification reaction was performed with preliminary denaturation for 10 min at 95°C, followed by 60 amplification cycles (with a temperature transition rate of 20°C/s) of denaturation at 95°C for 5 s, touchdown annealing from 60°C to 57°C for 10 s (temperature step size of 0.2°C), and primer extension at 72°C for 6 s, with a single fluorescence acquisition step at the end of extension. A final cooling step was performed at 40°C for 10 s. Light emission was monitored through the F1 channel of the instrument. Crossing points (Cp) were determined by the second derivative function. The data were analyzed by using Roche LightCycler software version 3.5.3.

The 20-μl reaction mixture for *H. pylori*-specific 23S rRNA gene amplification (121-bp PCR fragment) and melting peak analysis was prepared as follows: 2 μl of LightCycler-FastStart DNA Master SYBR Green I (Roche Molecular Biochemicals), 4 mM $MgCl_2$, 0.075 μM primer 23S-F (5′-AGATGGGAGCTGTC TCAACCAG-3′, positions 2437 to 2458, GenBank accession no. U27270), 0.25 μM primer 23S-R (5′-TCCTGCGCATGATATTCCC-3′, positions 2573 to 2555, GenBank accession no. U27270), 0.2 μM probe 23S-S (5′-Cy5-AAGACGGAA AGACCCCGT-biotin-3′, positions 2507 to 2524, GenBank accession no. U27270), and 2 μl of DNA extract made up to 20 μl with water. The reaction was performed with preliminary denaturation for 10 min at 95°C, followed by 70 amplification cycles (with a temperature transition rate of 20°C/s) of denaturation at 95°C for 5 s, annealing at 65°C for 10 s, and primer extension at 72°C for 6 s, with a single fluorescence acquisition step at the end of extension. This was followed by melting point analysis of the probe-PCR product duplex consisting of 95°C for 0 s, followed by cooling to 40°C for 60 s, before the temperature was raised to 95°C at a rate of 0.2°C/s with continuous fluorescence acquisition. A final cooling step was performed at 40°C for 10 s. Light emission was monitored through the F1 channel of the instrument, and Cp values were determined by the second derivative function. Melting curves were constructed automatically by the Roche LightCycler software version 3.5.3 and were analyzed in the F3 channel of the instrument. Samples were considered to be *H. pylori* positive upon determination of a biprobe-specific melting curve.

In order to evaluate the detection limit of both PCR assays, dilutions of *H. pylori* DNA ranging from 5,000 to 1 fg were used as a template DNA. Furthermore, 0.2 g stool of two noninfected individuals were spiked with dilutions of *H. pylori* DNA in a range from 5,000 fg to 200 fg and analyzed as described above. The efficiency of both assays was determined by standard curves on the basis of 10-fold serial dilutions of *H. pylori* DNA. *H. pylori* specificity of both PCR assays was examined by using bacterial DNAs of other *Helicobacter* and *Campylobacter* species, e.g., *H. hepaticus* (ATCC 51448), *H. bilis* (ATCC 51630), *H. pullorum* (clinical isolate), *H. rappini* (ATCC 43879), and *C. jejuni* (clinical isolate).

The melting point of the probe-23S rRNA PCR product duplex was determined with DNA of the clarithromycin-susceptible (wild-type) strain CCUG 38771 and of the clarithromycin-resistant (mutant) strains 825, 683, and 677, each harboring one of the three possible point mutations responsible for clarithromycin resistance. In order to evaluate the sensitivity of detecting mutant genotypes among wild-type genotypes in the presence of both, A2143G mutant DNA (strain 677) was mixed with wild-type DNA at ratios of 1:1, 1:5, 1:10, 1:15, and 1:20. Mixtures were tested by 23S rRNA real-time PCR and melting curve analysis.

Statistical analysis. Comparisons of Cp values between biopsy and stool samples for each of the two PCR assays, as well as between the two assays either for biopsy or stool samples, were performed by paired *t* test, and coefficients of correlation (*R*) were determined by using SPSS 10.0 for Windows. A *P* value of <0.05 was considered statistically significant. Specificity, sensitivity, and positive and negative predictive values (PPVs and NPVs, respectively) were calculated by chi-square test.

RESULTS

Performance of PCR. For both real-time PCR assays, the lowest pure *H. pylori* DNA concentration always delivering a positive result was 4 fg per PCR, which is equivalent to 2.2 bacteria, if estimated that one bacterium corresponds to ca. 1.8 fg of DNA by a genome size of 1,667,867 bp (21). Tenfold serial dilutions of purified *H. pylori* DNA in the range from 4 ng to 4 fg revealed Cp values of the *ureA*/23S rRNA gene assays of 19.7/17.6, 23.3/20.6, 27.1/24.5, 30.8/28.2, 34.5/32.2, 37.9/35.9, and 42.7/41.1. Since samples were run in duplicate, Cp values represent the mean of two individual reactions. In a range between 4 ng and 40 fg, a slope of 3.7 was obtained with both standard curves, indicating a highly efficient amplification. As expected, Cp values were inconsistent at a DNA concentration as low as 4 fg per reaction and varied for the *ureA* assay between 41.1 and 46.3 and for the 23S rRNA gene assay between 40.4 and 43.7. In two negative stool specimens inoculated with *H. pylori* DNA prior to DNA extraction the detection limit for both assays was 10 to 15 pg/g stool, which is

TABLE 1. Comparison of culture and clarithromycin susceptibility testing by E-test to the results obtained by real-time PCR (*ureA* and 23S rRNA gene) in biopsy and stool samples of 92 patients

No. of patients (no. of patients who received eradication therapy)	Biopsy				Stool (real-time PCR)	
	Culture		Real-time PCR			
	Growth	E-test	*ureA*[c]	23S rRNA[c]	*ureA*[d]	23S rRNA[c]
44 (10)	−	−	−	−	−	−
2[a]	−	−	+	S	−	−
1[b] (1)	−	−	+	R	+	R
1	+	S[e]	+	S	−	−
33 (2)	+	S	+	S	+	S
6 (5)	+	R[f]	+	R	+	R
1	+	R	+	R+S	+	R+S
2	+	R	+	R+S	+	S
1	+	R	+	S	+	R+S
1	+	R	+	S	+	S

[a] Histology negative, rapid urease test positive.
[b] Histology and rapid urease test negative.
[c] In three cases only one of the reactions run in duplicate was positive.
[d] In five cases only one of the reactions run in duplicate was positive.
[e] S, sensitive to clarithromycin.
[f] R, resistant to clarithromycin.

equivalent to 5.5 to 8.3 $\times$ 10^3 bacteria. At the detection limit, Cp values varied between 40.0 and 44.4 for the *ureA* assay and between 40.7 and 48.0 for the 23S rRNA gene assay. To prove the specificity of both PCR assays, bacterial DNAs of *H. hepaticus*, *H. bilis*, *H. pullorum*, *H. rappini*, and *C. jejuni* were tested by both assays. No amplification was observed with the *ureA* assay. The 23S rRNA assay was negative for *C. jejuni* and *H. pullorum* but positive for *H. hepaticus*, *H. bilis*, and *H. rappini*, with a detection limit of 40 fg of pure bacterial DNA per PCR and with an amplification delay of 10 cycles compared to the same amount of *H. pylori* DNA. However, no amplification was observed when 1 g of *H. pylori*-negative stool was inoculated with up to 3,000 pg of *H. hepaticus*, *H. bilis*, or *H. rappini* DNA (an equivalent of 1.6 $\times$ 10^6 bacteria).

Detection of *H. pylori* infection by PCR. With regard to the clinical evaluation of both tests, 45 of the 92 patients were shown to be infected. They were all found to be positive by culture and positive also by both real-time PCR assays when biopsies were used (Table 1). From all except one culture-positive patient, the 23S rRNA and *ureA* genes could be amplified successfully from stool (Table 1). Only a few colonies were grown in the biopsy culture of this patient, indicating a low *H. pylori* density. This also correlates with the biopsy PCR results, since the Cp values of 38 as determined by the *ureA* assay and 39 as determined by the 23S rRNA gene assay were shown to be in the upper range. Two patients were found to be negative by culture and histology but positive by rapid urease test. These patients were positive by both real-time PCR assays in their biopsies but not in their stool samples (Table 1). Since they did not fulfill the criteria for a definite characterization of their *H. pylori* status, they were not considered for statistical evaluations. Another patient was culture, histology, and rapid urease test negative; however, the biopsy and stool samples were positive by both real-time PCR assays (Table 1).

Overall, as determined by the *ureA* assay the Cp values were 24 to 40 for biopsies and 33 to 41 for stool specimens, whereas as determined by the 23S rRNA gene assay the Cp values ranged 23 to 39 and 33 to 46 for biopsies and stool specimens, respectively. A comparison of Cp values obtained by the *ureA* assay to those obtained by the 23S rRNA gene assay for both biopsy and stool specimens is shown in Fig. 1A and B and revealed coefficients of correlation of 0.83 and 0.52, respectively ($P < 0.001$). Comparisons of Cp values between biopsy and stool samples for each of the two PCR assays are shown in Fig. 1C and D. The coefficients of correlation with the *ureA* and the 23S rRNA gene assays were 0.33 and 0.39, respectively ($P < 0.05$).

The remaining 44 patients were not *H. pylori* infected, and their biopsy and stool samples were all negative by both real-time PCR assays. Specificities, sensitivities, PPVs, and NPVs of real-time PCR assays for both biopsies and stool samples are presented in Table 2. With respect to the detection of *H. pylori* infection, the two assays delivered identical results.

Clarithromycin susceptibility testing by PCR. 23S rRNA PCR and melting curve analysis with DNA extracts of *H. pylori* control strains produced melting curves with melting temperatures of 63°C for the wild type, 58°C for the A2142C mutant, and 54°C for the A2142G and A2143G mutants. No shift in the melting temperature was observed when *H. pylori*-negative biopsy homogenates or stool samples were inoculated with the DNA extract of those strains. Figure 2 shows the melting peaks obtained from stool DNA extracts of patients infected with a clarithromycin-sensitive (wild-type) and/or -resistant (mutant) genotype.

Of the 45 culture-positive patients, 34 were infected with a clarithromycin-susceptible strain and 11 (24.4%) with a clarithromycin-resistant strain (Table 1). All cases with a clarithromycin-susceptible strain were also confirmed by real-time PCR in both biopsy and stool samples, with the exception of one stool sample since no amplification product was obtained (Table 1).

Of the 11 cases with a resistant strain, the resistant genotype was confirmed by real-time PCR in both biopsy and stool samples in seven cases, whereas in one of these cases the susceptible genotype could also be detected (Table 1). In two cases, both the susceptible and the resistant genotypes were detected in the biopsies, whereas in stool only the susceptible genotype could be detected (Table 1). In another case, the susceptible genotype was detected in both stool and biopsy, whereas the resistant genotype was detected only in stool (Table 1). Finally, in one case only the susceptible genotype could be detected in both stool and biopsy (Table 1). The sensitivities, specificities, PPVs and NPVs of clarithromycin susceptibility testing by real-time PCR are shown in Table 2.

Thus, in 4 of 11 cases with a resistant strain as shown by the E-test, real-time PCR results suggest the presence of both wild-type and mutant genotypes. These cases and the one case in which PCR failed to detect the resistant genotype were further investigated also in order to exclude the occurrence of some other point mutations in the 23S rRNA gene which our assay might not detect. Clarithromycin susceptibility testing of the corresponding isolates was repeated by E-test. Although numerous colonies showed clarithromycin MICs of >1 mg/liter, an inhibition ellipse, indicating the presence of a susceptible population, could also be observed. Bacteria were harvested both within and outside the inhibition ellipse, the DNA

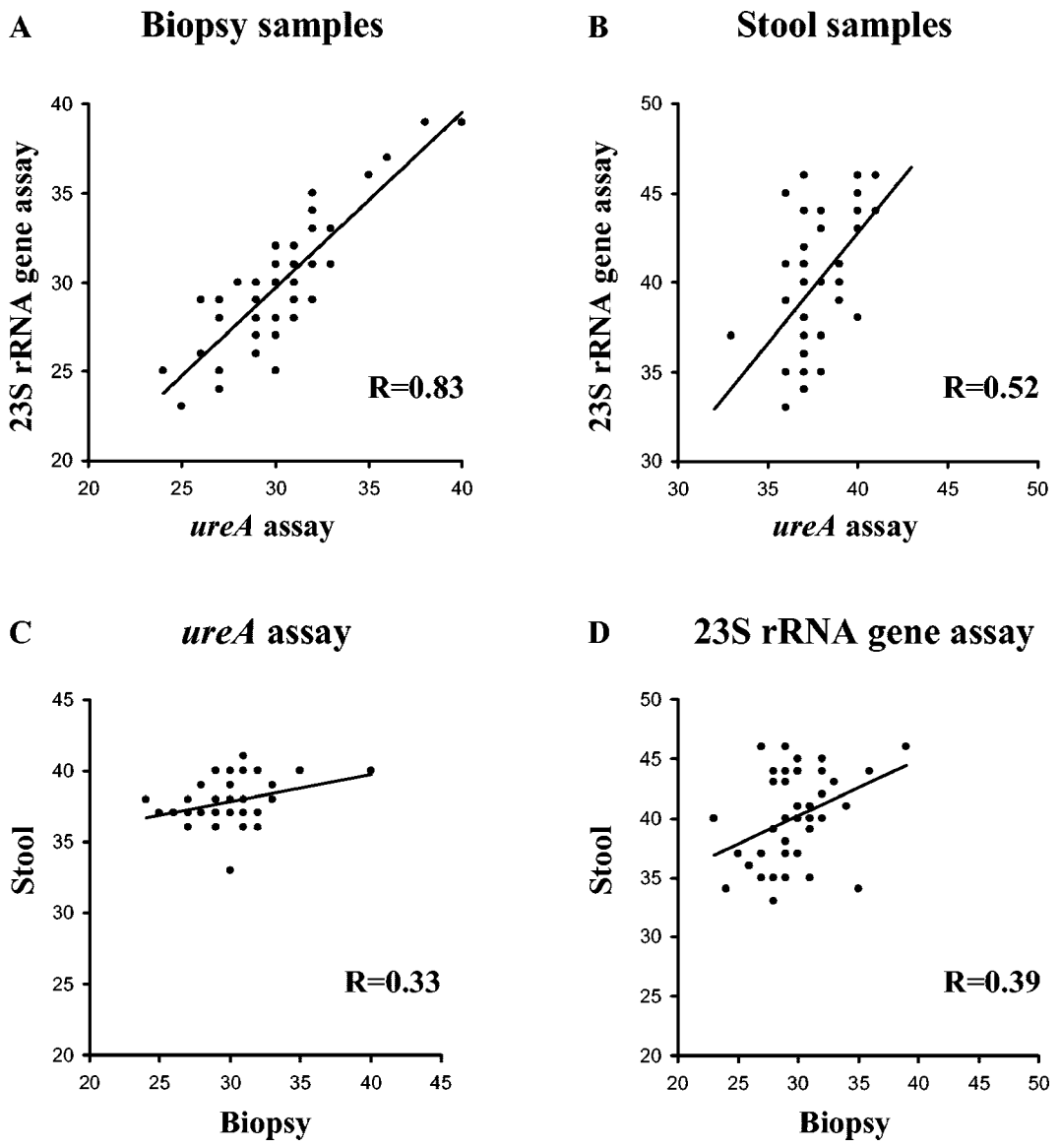

FIG. 1. (A and B) Comparison of Cp values obtained by the *ureA* assay to those obtained by the 23S rRNA gene assay for biopsies (A) and stool specimens (B). (C and D) Comparisons of Cp values between biopsy and stool samples for the *ureA* assay (C) and the 23S rRNA gene assay (D); three cases with positive biopsy but negative stool PCR results are not shown.

TABLE 2. Real-time PCR for detection of *H. pylori* infection (*ureA* and 23S rRNA gene assays) and clarithromycin susceptibility testing (23S rRNA gene assay): specificities, sensitivities, PPVs, and NPVs for biopsy and stool samples

Parameter	Detection of *H. pylori* infection		Clarithromycin susceptibility testing	
	Biopsy	Stool	Biopsy	Stool
Specificity (%)	98	98	100	100
Sensitivity (%)	100	98	82	73
PPV (%)	98	98	100	100
NPV (%)	100	98	94	92

was extracted, and real-time PCR was performed. Within the inhibition ellipse, melting curve analysis revealed the A2142/43G mutant genotypes, whereas outside the ellipse either both wild-type and mutant genotypes or only the susceptible wild-type genotype could be detected. In our study, only A-to-G transitions were detected in all cases of resistant strains, whereas the A-to-C transition was not found in any of the cases.

DISCUSSION

In recent years, several attempts have been made to detect *H. pylori* DNA in stool samples by conventional PCR (1, 6, 11, 12, 15, 19, 20). However, most of these reports have revealed low sensitivity. Nested PCR generally increases sensitivity but also has a high risk of contamination. Real-time PCR has

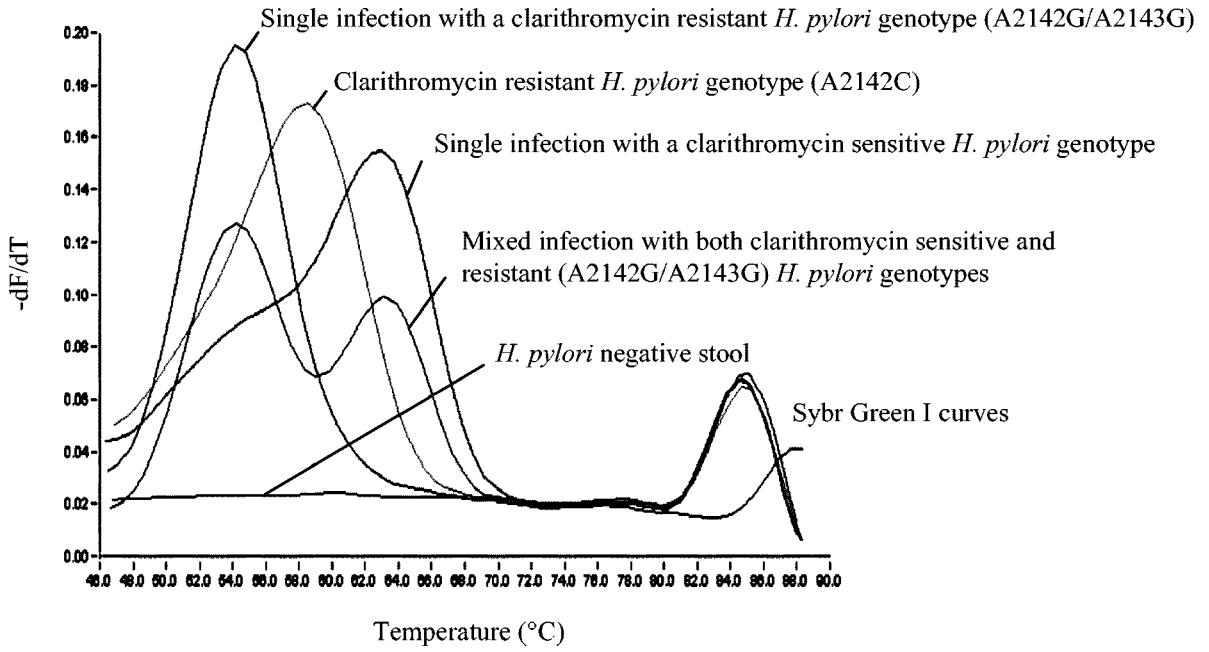

FIG. 2. Melting peaks obtained from stool DNA extracts of patients infected with a clarithromycin-sensitive (wild-type) and/or a clarithromycin-resistant (mutant) genotype. Sensitive genotypes showed a melting temperature of 63°C. Resistant genotypes with an A-to-G transition showed a melting temperature of 54°C. Mutants with an A-to-C transversion showed a melting temperature of 58°C (the curve was obtained from purified *H. pylori* DNA, since no such mutation was detected in the stool samples of patients). No biprobe-specific melting curves were obtained from noninfected patients. Values on the *y* axis are the first negative derivative of the change in fluorescence (dF) divided by the change in temperature (dT) (i.e., −dF/dT).

several advantages over conventional PCR, such as short working time, high specificity, and low risk of contamination. Thus far, two studies used real-time PCR targeting either *ureC* or 16S rRNA for the quantitative detection of *H. pylori* in gastric biopsies (7, 9). Most importantly, real-time PCR protocols on the basis of biprobes (2, 5) or hyprobes (10, 14, 18) have been developed during recent years, enabling the detection of the point mutations on the 23S rRNA which are associated with resistance to clarithromycin. These protocols were then used for clarithromycin susceptibility testing of cultured isolates or directly in the gastric biopsy and delivered accurate results.

As yet, no published data exist on the applicability of real-time PCR protocols for *H. pylori* detection and clarithromycin susceptibility testing in the stool samples of dyspeptic patients. However, such an application would be of great value, in particular considering the fact that primary care physicians mainly use noninvasive methods to diagnose *H. pylori* infection and treat patients by using first-line regimens without information on susceptibility to clarithromycin.

The biprobe-based real-time PCR assay developed here allows for the simultaneous detection of *H. pylori* and of point mutations in the 23S rRNA gene responsible for clarithromycin resistance. Preliminary investigations with stools of noninfected individuals inoculated with *H. pylori* DNA and, in particular, the clinical evaluation of this assay clearly demonstrated its suitability not only for biopsies but also for stool specimens.

H. pylori as a nonintestinal pathogen is present only in low numbers in feces and, therefore, the right choice of highly specific and sensitive primers is of crucial importance in order to obtain accurate results. However, the sequence homology of the 23S rRNA region between different *Helicobacter* species is high. For example, sequence comparisons between *H. pylori* and *H. hepaticus* revealed only three mismatches at the primer 23S-R annealing site. This seems to be the explanation for the observed cross-reactivity when amounts of >40 fg of pure *H. hepaticus*, *H. bilis*, or *H. rappini* DNA were subjected to the 23S rRNA PCR. On the other hand, this finding is very unlikely to be of any practical relevance, since inoculation of *H. pylori*-negative stool with large amounts of DNA from these species–equivalent to up to 1.6×10^6 bacteria/g stool—did not lead to a positive PCR result. Since *H. hepaticus*, *H. bilis* and *H. rappini* are also nonintestinal pathogens, it is highly unlikely that they can achieve concentrations as high as that in the bowel.

For a better estimation of the performance of the 23S rRNA gene assay with respect to both sensitivity and, in particular, specificity, a second highly specific real-time PCR test for the detection of *H. pylori ureA* was established. Considering the low copy number of target DNA in stool and the fact that stool represents a complex specimen comprising PCR inhibitors (which may considerably vary between specimens and/or patients), a relatively high number of amplification cycles was run by both assays to increase sensitivity. Thus, 70 amplification cycles were run by the 23S rRNA gene assay as reported previously (2). Since the highest Cp value ever measured in a positive stool sample was 46 in the present study, a lower cycle number may also have been appropriate. On the other hand, it should be mentioned that according to the biprobe protocol, as an endpoint analysis, only a probe-specific melting peak is considered to be a positive result. This melting peak is more

pronounced the more specific amplification product is present. This, however, occurs 10 cycles over the Cp value at the earliest. With regard to inhibition of the PCR, this was not observed either with biopsy or with stool samples when 2 μl of the DNA extract was added to the amplification reaction. The easy-to-handle QIAamp DNA stool minikit is thus likely to remove these substances to a considerable extent.

In any case, a high number of amplification cycles may lead to the buildup of nonspecific products. Analysis of PCR products by gel electrophoresis (not shown) revealed the presence of nonspecific amplicons in ca. 80% of negative biopsy and 40% of negative stool samples by the 23S rRNA gene assay. Since by this assay Cp values are due to the SybrGreen I light emission irrespective of whether a specific or a nonspecific double-stranded DNA is generated, the Cp values of these samples were in a range between 40 and 66. However, they all proved to be negative by endpoint hybridization melting point analysis. With respect to the *ureA* assay, nonspecific products were detected by gel electrophoresis in ca. 40% of negative biopsy and 60% of negative stool samples without affecting the specificity of the assay, since a positive signal during the real-time protocol is only dependent on the specific binding and cleavage of the TaqMan probe. Both PCR assays did not show any nonspecific amplification products in positive biopsy samples by gel electrophoresis analysis. In positive stool samples, however, additional nonspecific bands were also detected in almost 30% of the *ureA* assay PCR products, whereas analysis of the 23S rRNA gene assay PCR products revealed the specific band only. Although not influencing the accuracy of the *ureA* assay, the buildup of additional nonspecific products seemed to be associated with a shift to higher Cp values. Thus, the Cp values of the *ureA* assay were always lower in the absence but mostly higher in the presence of nonspecific amplification products than the corresponding Cp values of the 23S rRNA gene assay. Among other reasons, such as a patchy distribution of the target DNA at low concentrations, this may indeed at least partly explain the relatively poor correlation between the Cp values of the two assays in positive stool samples (Fig. 1B).

Poor results were also obtained for the correlation of the Cp values between biopsy and stool samples determined by each of the assays (Fig. 1C and D). Several reasons may be responsible for this finding. On the one hand, the bacterial load of two biopsies may not represent the actual bacterial density in the stomach. On the other hand, a number of factors, e.g., the velocity of the gastric epithelial turnover, bile production, alimentary factors, and the retention period in the bowel, may affect the amount of *H. pylori* DNA excreted in stool. Thus, Cp values of either biopsies or stool specimens by both assays may not correspond to the real bacterial density in the gastric mucosa. On the other hand, quantitation of *H. pylori* in biopsies or stool samples was not the goal of the present study since this is only of limited relevance in clinical practice.

The 23S rRNA gene real-time PCR was shown to be not only as sensitive but also as specific as the *ureA* assay, delivering identical results during clinical evaluation (Table 2). Both real-time PCR assays showed sensitivities and specificities comparable to that of the "gold standard" reference diagnostic methods: the rapid urease test, histology, and culture. As a matter of fact, it is possible that the two patients found to be positive by the rapid urease test and by both PCR assays in their biopsies were actually *H. pylori* infected and that low bacterial density and a patchy distribution of the pathogen may have been the reasons for the negative culture and histology results. This may also be true for the patient determined to be negative by the rapid urease test, histology, and culture but positive by both PCR assays in the biopsy and the stool specimen, thus resulting in a reduction of the PCR specificity to 98%.

In our study population, 11 of 45 culture positive patients (24.4%) were infected by a resistant strain as determined by E-test. This finding is in contrast to the findings of Fontana et al. (4), who performed clarithromycin susceptibility testing with stool specimens by seminested PCR and restriction fragment length polymorphism analysis. In that study, only 2 of 125 *H. pylori*-positive patients (1.6%) were shown to be infected by a clarithromycin-resistant strain, and in both cases a T2717C mutation was detected.

In the present study, the specificity of the 23S rRNA PCR with respect to detection of resistance to clarithromycin was 100% in both biopsies and stool specimens. However, the sensitivity of PCR was 82% in biopsies and 73% in stool specimens since PCR failed to detect the resistant genotype in two and three cases, respectively. In four cases, PCR revealed both the susceptible and the resistant genotypes, whereas in one case it failed to detect the resistant genotype at all (Table 1). However, reexamination of the corresponding isolates by E-test and subsequent analysis of the bacterial populations within and outside the inhibition ellipse by PCR showed the presence of both genotypes in all five cases. Thus, up to a certain ratio the susceptible genotype is likely to suppress the amplification of the mutant genotype. This was also found in preliminary experiments, where mixtures of DNA of the wild-type and the A2143G mutant up to a ratio of 10:1 (i.e., wild type to A2143G mutant) resulted in the detection of only the wild-type genotype by real-time PCR. This observation was also made by others, who investigated clarithromycin resistance in biopsies by hyprobe-based 23S rRNA real-time PCR (14, 18).

Interestingly, 5 of 45 individuals (11.1%) in our study population were infected by both the susceptible and the resistant genotypes. All of these patients had not been treated for *H. pylori* infection previously. On the other hand, five of the six patients in whom infection by only a resistant genotype was identified by real-time PCR had received eradication therapy consisting of a combination of a clarithromycin, amoxicillin, and a proton pump inhibitor in the past (Table 1). As expected, the resistant genotype was selected during treatment in these patients.

In conclusion, a 23S rRNA real-time PCR assay was developed allowing, in combination with melting curve analysis, for the accurate detection of *H. pylori* infection and for clarithromycin susceptibility testing not only in biopsy but also in stool samples. In clinical practice where the "test (by noninvasive methods) and treat strategy" for dyspeptic patients still accounts for the majority of eradication attempts, this test may be of great value in particular when the emerging problem of clarithromycin resistant strains is being considered. Furthermore, this test seems to perfectly meet the requirements of a noninvasive assay for a posttreatment follow-up examination.

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